IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:

YUCH PING HSIEH et al.

Serial No. **09/990,783**

Filing Date: **11/14/2001**

For: **MICRORESPIROMETER AND ASSOCIATED METHODS**

Examiner: **Yelena G. Gakh**

Art Unit: **1743**

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AFFIDAVIT UNDER 37 CFR §1.132

STATE OF Florida
COUNTY OF Leon

Before me, the undersigned authority, personally appeared Yuch Ping Hsieh, Ph.D., who after first being duly sworn on oath, deposes and says as follows:

1. My full name is Yuch Ping Hsieh. I am Professor of Wetland Ecology at Florida A&M University. I hold a Ph.D. from Rutgers University in 1976, and have been Florida for 24 years.

2. I am over the age of 21 years and make this affidavit based upon my personal knowledge.

3. I am the first-named inventor on the above-referenced patent application.

4. In an Office Action dated October 4, 2005, the Examiner has objected to the Specification on the basis of 35 USC 112 as being nonenabling of the invention.

5. The experiments for optimizing operating conditions were performed with a glove box having a known CO_2 concentration because we need to know how alkaline solution absorb CO_2 under various CO_2 concentrations. The experiments presented in FIG. 6 were with real samples of various respiration rates (various bacteria activities from fresh meat to stale milk) because there are validations of the microrespirometer method with a proven method.

6. The Examiner has also questioned the data of FIG. 4. The relationship depicted in FIG. 4 is not obvious because it applies exclusively to very dilute alkaline solutions of less than 0.01 M. The relationship depicted in FIG. 4 was discovered as a result of FIG. 2, which illustrates that the CO_2 absorption rate (slope of the curves) by an alkaline solution depends on both the concentration of the alkaline solution and the concentration of CO_2 in the head space until the alkaline solution is below 0.01 M. When the alkaline solution is < 0.01 M, the CO_2 absorption rate depends on only the concentration of CO_2. FIG. 4 illustrates that a steady-state head-space CO_2 concentration can be established if there is a source (respiration) and a sink (diluted alkaline solution < 0.01 M) of CO_2 in an enclosed head space. That is, the respiration and CO_2 absorption of the diluted alkaline solution will adjust by itself to a steady-state CO_2 concentration in the head space as long the respiration does not change significantly and the alkaline solution is not completely neutralized. The steady-state CO_2 concentration in the head space is controlled by the respiration rate; i.e., the higher respiration rate, the higher the steady-state CO_2 concentration will be. The respiration rate then can be determined by simply measuring the CO_2 absorption rate of the alkaline solution at the steady state. Only under such a dilute alkaline concentration can the respiration rate be determined at the

μL/h level and within a short period of time (< 1 h). This measurement of respiration rate through steady-state CO_2 concentration in the head space is unique in this invention and is therefore respectfully believed to be patentable.

7. The Examiner has cited Stotzky (1965) as rendering Claims 1-13 unpatentable. Stotzky teaches the principle of exhaustive CO_2 absorption by alkaline solution to determine respiration rates. Stotzky's methods require alkaline concentrations greater than 0.3 M to exhaustively (completely) absorb CO_2 in the head space in order to determine respiration rate. The principle of exhaustive CO_2 absorption is completely different from the principle of establishing steady-state CO_2 concentration. No steady-state head space CO_2 concentration can be established by the Stotzky method because it would approach zero as the incubation is prolonged. The methods described by Stotzky have much higher detection limit of CO_2 (500 μL) than that of the present microrespirometer method (1 μL) because of the much higher alkaline concentration employed by Stotzky.

8. Further, the method described by Stotzky requires complete (exhaustive) absorption of head space CO_2. That is why it must use a much stronger alkaline solution (> 0.3 M) to exhaustively absorb the CO_2. Any substantial CO_2 remaining in the head space of the Stotzky methods contributes error. There is no mention of establishing steady-state CO_2 concentration in the head space by Stotzky because it should not and cannot. The "steady-state equilibrium" described in Stotzky (1965) is referring to the respiration of a soil sample that is established by the conditions of water content, temperature, aeration rate, and spatial arrangement of a soil sample (please see the discussion in p. 1553 of the Stotzky paper). The "steady state" of the microrespirometer is referring to the CO_2 concentration in the head space that is established by the balance

of CO_2 evolution (respiration) and CO_2 absorption of the diluted alkaline solution. The principles of this invention and that of Stotzky's are fundamentally different. The acid used in the titration of the microrespirometer method is the CO_2 respired by the sample rather than by a HCl solution as described by Stotzky. A comparison of the microrespirometer method and that of the Stotzky can be summarized in the following table:

Method	Principle of Operation	Concentration of Alkaline Solution	Detection Limit
Stotzky (1965)	***Completely*** and ***exhaustively absorption*** of the CO_2 from a continuous ***air stream*** or in an enclosed head space. >0.3 M alkaline solution is required in order to completely absorb the head space CO_2.	0.5-1 M NaOH	~500 µL in CO_2 level and >6 hr. in time.
This invention (micro-respirometer)	Establishing a steady-state CO_2 concentration in the ***enclosed head space*** during pre-incubation using ***very diluted*** alkaline solution. The CO_2 absorption rate of the alkaline solution is determined at the steady state (absorption rate = respiration rate). It is ***not an exhaustive CO_2 absorption*** method because certain CO_2 concentration is maintained in the head space throughout the determination.	1-5 mM NaOH	~1 µL in CO_2 level and < 1 hr. in time

9. The method described in the Specification and claimed in the present application has received the acknowledgment of peer review in the attached publications by one or more of the inventors. Exhibit A is a published paper entitled "Determination of Carbon Dioxide Evolution Rates Using a Novel Noninstrumental Microrespirometer," by Y. Hsieh and Y. Hsieh (*J. AOAC International* **83**(2), 277-281, 2000). The method was used to provide data for Exhibits B and C, also published in peer-reviewed journals: "Real-Time Determination of Microbial Activity of Pasteurized Fluid Milk Using a Novel Microrespirometer Method," Z. Ren and Y. Hsieh (*J. AOAC International* **88**(6), 1-6, 2005) and "Comparison of the Real-time Microrespirometer and

Aerobic Plate Count Methods for Determination of Microbial Quality in Ground Beef," X. Li and Y. Hsieh (*J. Food Science* **68**(9), 2758-63, 2003).

10. Therefore, I believe that the Specification is enabling, and that the claims patentably define over the cited reference of Stotzky.

FURTHER AFFIANT SAYETH NOT.

YUCH PING HSIEH

Subscribed and sworn to before me this 2nd day of March, 2006.

Notary Public
My commission expires:

Personally known to me _____
or
Produced identification of:

Kathleen D Cook/FDL

Ethel L. Bell
Commission # DD433432
Expires May 24, 2009
Bonded Troy Fain - Insurance, Inc 800-385-7019

EXHIBIT A

ENVIRONMENTAL ANALYSIS

Determination of Carbon Dioxide Evolution Rates Using a Novel Noninstrumental Microrespirometer

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A novel noninstrumental microrespirometer was developed to determine carbon dioxide evolution rates of solid or liquid samples at μL/h levels accurately and rapidly. The respirometer is based on the simple principle of acid–base titration at a steady-state of carbon dioxide absorption/evolution. The structure and operation of the microrespirometer are simple and the cost is modest in comparison to instrumental methods. The microrespirometer is suitable for laboratory studies and field routine examinations of food, agricultural, and environmental samples.

Respiration is a common indicator of biological activity. Respirometry (measurement of respiration rates) has been applied to a broad spectrum of applied and environmental microbiology, such as toxicity–treatability, process control and prediction of biological oxygen demand (BOD_5) in wastewater treatments (1, 2), assessment of metal toxicity (3), living soil microbial biomass (4–6), and food quality (7, 8).

Respiration rates can be measured either by rates of oxygen consumption or CO_2 evolution. Rapid oxygen consumption rate can be measured by using an oxygen probe or a quantitative electrolytic cell (2). Most oxygen respirometers, however, are applicable only to liquid samples. Oxygen respirometers with an electrolytic cell can be used to determine respiration of solid or semi-solid samples, but their sensitivity is greatly compromised.

Sensitive and rapid CO_2 respirometers based on infrared (IR) detectors have been developed in the last 3 decades (9), and can handle solid samples with high speed and sensitivity. Instrumental respirometers are technically complicated and expensive if accuracy and sensitivity are demanded. Noninstrumental CO_2 respirometers operated by an alkaline trap and acid–base titration have been in existence for years (10). They are simple but relatively slow (measurement in days) and less sensitive (detection limit in mL CO_2/day). Sensitive and rapid determination of respiration rates is highly desirable in monitoring microbial activity in food and environmental samples.

We developed a novel non-instrumental microrespirometer capable of determining CO_2 evolution rates of solid or liquid samples rapidly (in 1 h) and sensitively (μL/h level). The operation is simple and the cost is very modest. The microrespirometer can be conveniently operated under laboratory or field conditions.

Experimental

Materials

(a) *Microrespirometer.*—The design of the microrespirometer is based on the simple principle of acid–base titration between an alkaline solution and CO_2, with phenolphthalein to indicate the end point. The microresprometer consists of a transparent reaction chamber and a sample vial (Figure 1). The reaction chamber is, in fact, a small alkaline trap (total headspace, 6–7 mL) with a septum hole. The size of a sample vial may vary depending on the need (vials of 25, 30, 40, and 75 mL are available; e.g., Fisherbrand [Suwanee, GA] EPA bottles). The reaction chamber and the sample vial are coupled through a standard threaded screw and septum liner to form a closed headspace. Alkaline solution can be injected into the reaction chamber with a syringe. The alkaline solution absorbs the CO_2 in the headspace. The indicator in the alkaline solution changes from a deep to a faint pink when the alkaline solution is consumed by CO_2. The microrespirometer is shaken at a fixed rate of ca 240 rpm on an orbital shaker to enhance the absorption of CO_2.

(b) *Alkaline solution.*—The alkaline NaOH–$BaCl_2$–indicator solution contains an equal molar ratio of NaOH and $BaCl_2$ and 0.5 mL indicator solution (0.5% phenolphthalein in 50% ethanol solution) per 50 mL alkaline solution. $BaCl_2$ in the alkaline solution precipitates the absorbed CO_2 that ensures the stoichiometry of 2 moles of alkaline spent per mole of CO_2 absorbed, i.e.,

$$CO_2 + 2NaOH + BaCl_2 = BaCO_3(s) + 2NaCl \quad (1)$$

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[1] Author to whom correspondence should be addressed.

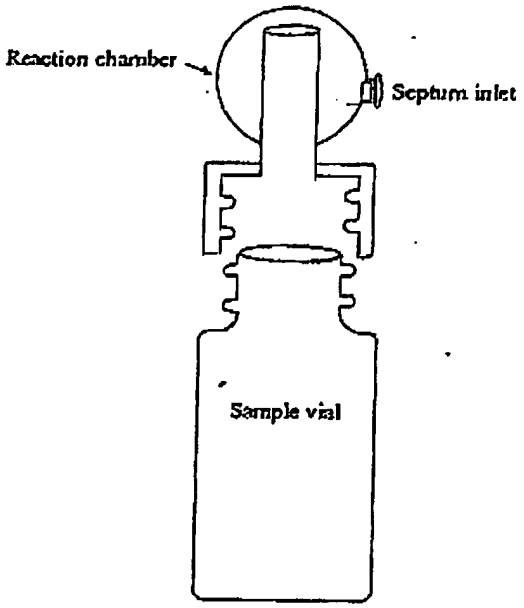

Figure 1. A graphic representation of the microrespirometer. The reaction chamber has a total headspace of 6–7 mL in which up to 1.2 mL can be used to hold the alkaline indicator solution. The size of the sample vial can be varied according to the test. Currently, 25, 40, and 75 mL sample vials are available commercially.

$BaCl_2$ also sharpens the change of color at the end point when a very low level of respiration is being determined. The alkaline solution is stored in a septum-capped vial to prevent absorption of CO_2 from the air. The alkaline solution is transferred through a syringe.

Procedures

The following experiments were performed to determine the optimal conditions for operating the microrespirometer:

(a) *Shaking rate experiment.*—The effect of shaking on the CO_2 absorption of the microrespirometer was investigated. Microrespirometers with empty 25 mL sample vials were coupled in a glove box of known CO_2 concentration (determined by an IR CO_2 analyzer). A 0.2 mL portion of 0.002M alkaline solution was injected into each reaction chamber. The microrespirometers were shaken at a fixed rate of 100, 150, 200, 250, or 300 rpm. The time required to consume the alkaline solution in each microrespirometer (as indicated by the color change) was recorded. Each treatment was performed in triplicate.

(b) *Alkaline concentration experiment.*—The effect of alkaline concentration on the absorption of CO_2 in a closed headspace was investigated. A 25 mL sample vial was connected to an IR analyzer as described by Hsieh and Hsieh (9) so that the vial and the IR detector formed a closed headspace in which air circulated continuously. The 25 mL vial was shaken at 240 rpm on an orbital shaker. A 1 mL portion of 0.2, 0.1, 0.01, or 0.001M alkaline solution was injected into the vial through a syringe needle port at the beginning of the experiment and the concentration of CO_2 in the vial was recorded periodically. The experiment was repeated twice.

(c) *CO_2 concentration experiment.*—The relationship between the CO_2 absorption rate and the CO_2 concentration in the headspace of the microrespirometer was investigated. Microrespirometers with a 75 mL sample vial were coupled in a glove box of known CO_2 concentration. An increment of 0.1 mL 0.002M alkaline solution was injected into the reaction chamber. The microrespirometers were shaken at 240 rpm and the time required to consume each increment of the alkaline solution was recorded. The consumption of each increment of the alkaline solution (i.e., 0.2 μmol alkaline, or 0.1 μmol CO_2) represents a 29.7 ppm (v/v) reduction of CO_2 concentration in the 82 mL microrespirometer at 25°C. Each treatment was performed in triplicate.

(d) *Microrespirometer procedure.*—A portion of solid or liquid sample was place in a sample vial and the vial was coupled to a reaction chamber. 0.8 mL alkaline solution of suitable concentration was injected into the reaction chamber using a syringe. The respirometer was shaken at a fixed rate of ca 240 rpm for 30 min (the pre-equilibration period), ensuring that the alkaline solution was not completely consumed during this time. If the alkaline solution wass about to be consumed, more alkaline solution was injected into the reaction chamber. After 30 min pre-equilibration, the shaker was stopped and the alkaline solution in the reaction chamber was

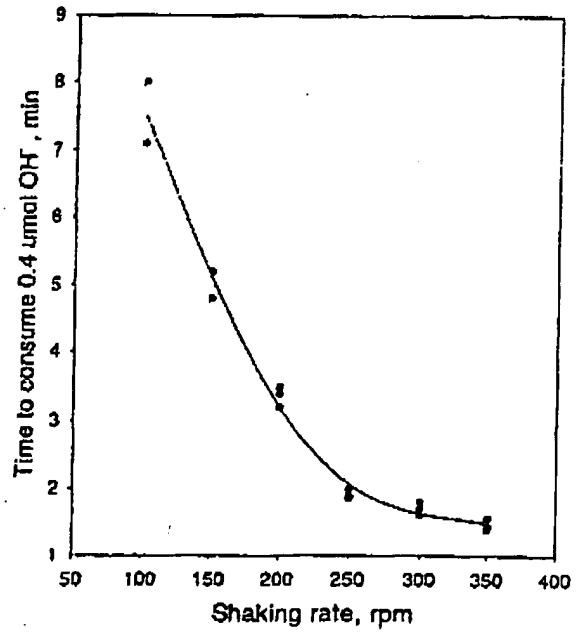

Figure 2. Relationship between CO_2 absorption and shaking rate of the microrespirometer at 25°C. Each dot represents a single measurement, not a mean.

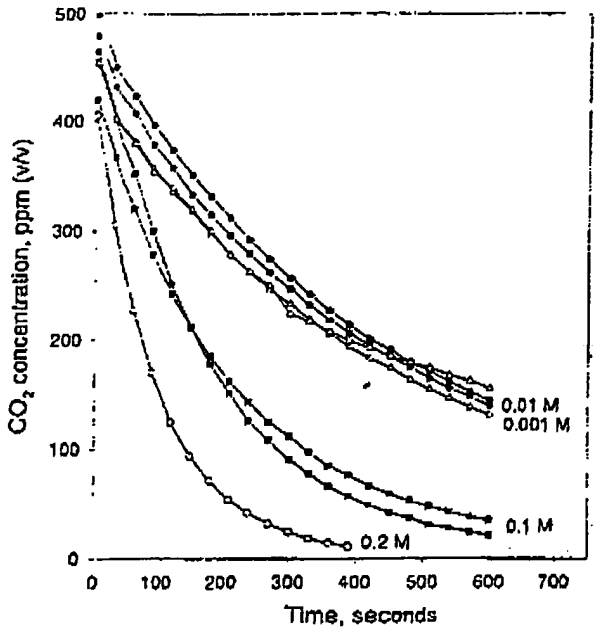

Figure 3. Relationship between CO_2 absorption and concentration of the alkaline solution in the microrespirometer. The experiment was performed at 25°C and a shaking rate of 240 rpm. Each dot represents a single measurement, not a mean.

withdrawn to ca 0.1–0.2 mL remaining. The respirometer continued to be shaken until the alkaline solution changed to a faint pink. The shaker was stopped immediately,and 0.1 mL alkaline solution was injected, shaking resumed, and the time required to consume the alkalinity was recorded. Increments of 0.1 mL alkaline solution were injected twice more and the time required to consume each increment was recorded. The average of the time required to consume the 0.1 mL increment alkaline solution was used to calculate the CO_2 evolution rate with the following formula:

$$CO_2 \text{ evolution rate, } \mu mol/h = \frac{0.1 \times 10^3 \times M/2}{t/60} \quad (2)$$

where M is the concentration of the alkaline solution in mol/L and t is the time required to consume 0.1 mL alkaline solution in min. The CO_2 evolution rate can be expressed in μL/h by multiplying the molar volume of CO_2 at a specific temperature.

(e) *Validation experiment.*—The CO_2 evolution rates determined by the microrespirometer method were compared with those determined by an established IR analyzer method. Portions of soil samples of relatively low CO_2 evolution rates (2–5 μL/h/g), unfrozen processed meat samples of medium CO_2 evolution rates (10–100 μL/h/5 g), and room temperature milk samples of high CO_2 evolution rate (80–280 μL/h/20 mL) were placed in 25 mL sample vials. The CO_2 evolved by microorganisms associated with each sample was determined by the microrespirometer method. A duplicate sample in another 25 mL sample vial was also placed in a 250 mL flask and the CO_2 evolution rate was determined by the IR analyzer method described by Hsieh and Hsieh (9). The sample vials in the microrespirometers and those in the 250 mL flasks of the IR analyzer were exchanged and the CO_2 rates were determined again with the alternative methods.

Results and Discussion

The effect of shaking rates on CO_2 absorption of the microrespirometer is shown in Figure 2. The CO_2 absorption increased as the shaking rate increased from 100 to 250 rpm. The increase in CO_2 absorption leveled off when the shaking rate exceeded 250 rpm. Shaking at 200 rpm or higher improved reproducibility of CO_2 absorption. A fixed shaking rate between 200 and 250 rpm is recommended for the microrespirometer because the benefit of shaking is achieved while the difficulty of operation at higher rates is avoided.

The results of the alkaline concentration experiment are presented in Figure 3. As the concentration of alkaline solution decreased from 0.2 to 0.01M, the CO_2 absorption rate decreased as well. The CO_2 absorption rate did not further decrease as the alkaline concentration was reduced from 0.01 to 0.001M. It is not possible to have complete absorption of CO_2 in the headspace of a microrespirometer in a matter of hours when the concentration of the alkaline solution is less than 0.01M. The concentration of the alkaline solution has to be

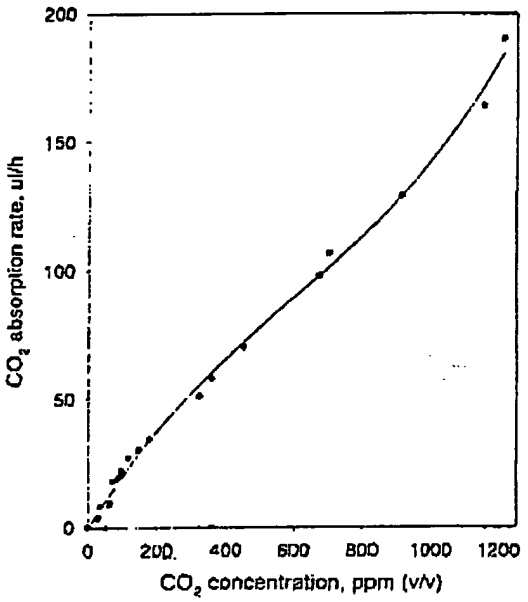

Figure 4. Relationship between CO_2 absorption and concentration of CO_2 in the headspace (82 mL) of the microrespirometer. The experiment was performed at 25°C and a shaking rate of 240 rpm. Each dot represents a single measurement, not a mean.

much less than 0.01M in order to determine CO_2 evolution rate at a μL/h level. The microrespirometer, therefore, does not work on the principle of a complete CO_2 absorption but on an absorption/evolution equilibrium principle that will be discussed in detail later. An alkaline solution of less than 0.0005M is not stable enough to be used in the microrespirometer because the possibility of contamination from the ambient CO_2 is too large for such low alkalinity. Phenolphthalein is not stable in alkaline concentrations exceeding 0.01M; the deep pink color will fade away by itself within 1 h. Therefore, a proper alkaline concentration range suitable for the microrespirometer is between 0.01 and 0.001M.

Figure 4 depicts the relationships between the CO_2 absorption rate of a 0.002M alkaline solution and the concentration of the CO_2 in the headspace of a microrespirometer. In general, the CO_2 absorption rate has a positive curve–linear relationship with the concentration of CO_2. The CO_2 absorption rate of the respirometer at a given temperature and shaking rate reflects the CO_2 concentration in the headspace of the microrespirometer which is, of course, not necessarily the CO_2 evolution rate of the sample. However, if a sample is equilibrated with the alkaline solution in the respirometer at a given temperature and a given shaking rate, then the concentration of CO_2 in the respirometer would eventually reach a constant value when the CO_2 absorption rate equals the CO_2 evolution rate. For example, if the beginning CO_2 concentration of the respirometer is 450 ppm and the CO_2 evolution rate of the sample is 100 μL/h, the CO_2 concentration of the respirometer will be increased to about 660 ppm (Figure 4) and stay there because an equilibrium of CO_2 absorption and evolution is established. If the CO_2 evolution rate of the sample is 20 μL/h, the CO_2 concentration of the respirometer will be decreased to about 150 ppm (Figure 4), where an absorption/evolution equilibrium is established. The CO_2 evolution rate of a sample, therefore, can be determined by the CO_2 absorption rate of the microrespirometer when an equilibrium or steady-state is established. That is, after a sample is equilibrated with an alkaline solution in a microrespirometer, the CO_2 evolution rate can be determined by the time required to consume a small increment of the alkaline solution (equation 2).

The minimum time required for a sample in the respirometer to reach an equilibrium was deduced from a computer simulation based on the relationship between the CO_2 absorption rate and the CO_2 concentration of the respirometer and the CO_2 evolution rate of the sample. That is, the concentration of CO_2 in the headspace of a respirometer after being shaken for a small increment of time Δt is,

$$C_{i+\Delta t} = C_i + \frac{(A_{Ci} - E)\Delta t}{V_{headspace}} \qquad (3)$$

where C_i and $C_{i+\Delta t}$ are the CO_2 concentrations of the respirometer at time i and time $i + \Delta t$, respectively. A_{Ci} is the CO_2 absorption rate of the respirometer at time i and a function of the CO_2 concentration C_i. E is the CO_2 evolution rate of the sample and $V_{headspace}$ is the volume of the headspace. The mathematical relationship of A_{Ci} and C_i was generated by a nonlinear regression curve fitting program (TableCurve, Jandel Scientific, San Rafael, CA) using the data of Figure 4. The regression enabled the calculation of A_{Ci} based on C_i. The values of A_{Ci}, C_i, and $C_{i+\Delta t}$ for each small time increment (0.5 min) of Δt were calculated and tabulated using a spreadsheet software (Excel, Microsoft, Redmond, WA) based on equation 3. An equilibrium is attained in the simulation when the CO_2 concentration in the respirometer approaches a constant, i.e., $(A_{Ci} - E)$ approaches 0 and $C_{i+\Delta t}$ approaches C_i. The minimum time required to attain an equilibrium is the sum of all small time increments, Δt, during which CO_2 concentration approaches a constant. The ratio of the CO_2 absorption rate to evolution rate (i.e., A_{Ci}/E) expressed as a percentage of the CO_2 evolution rate during the time course of reaching an equilibrium is presented in Figure 5. Two headspace volumes of the respirometer, i.e., 12 mL (5 mL remaining headspace in the sample vial plus 7 mL in the reaction chamber) and 27 mL (20 mL remaining headspace in the sample vial plus 7 mL in the reaction chamber) were simulated in Figure 5.

The results indicated that the smaller the headspace, the quicker an equilibrium is reached, and that the greater the CO_2

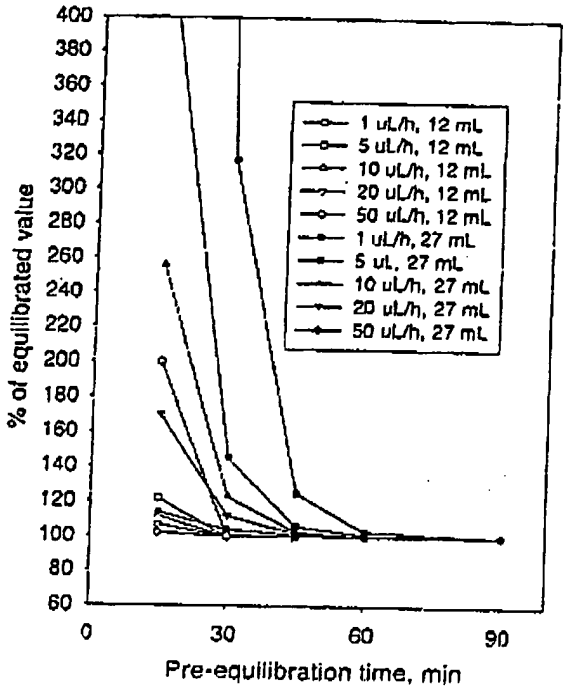

Figure 5. The relationship between the "pre-equilibration" time and the % of the equilibrated value (ratio of CO_2 absorption rate/equilibrated CO_2 absorption rate multiplied by 100) for a sample in a microrespirometer using 0.002M alkaline solution.

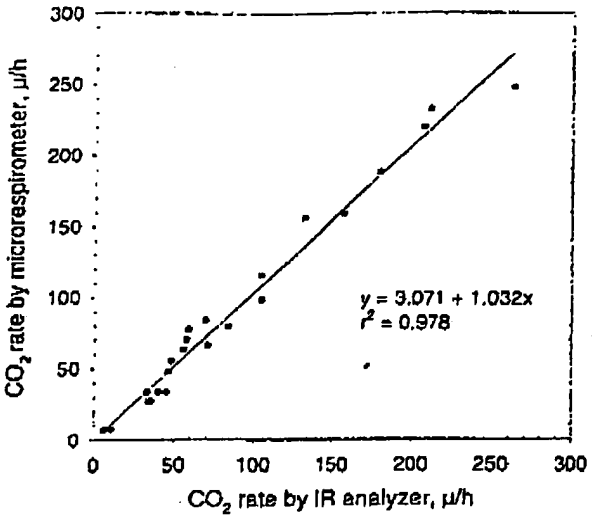

Figure 6. Comparison of the results of the CO_2 evolution rate determined by the microrespirometer with an established IR analyzer method. Each dot represents a single measurement, not a mean.

evolution rates, the quicker an equilibrium is reached. For example, in the 12 mL headspace case, 30 min pre-equilibration is sufficient for the measurement of all CO_2 evolution rates ≥ 1 µL/h. In the 27 mL headspace case, 100–107% of equilibrated value can be attained within 45 min for all CO_2 evolution rates, except the 1 µL/h case. The working range of the respirometer is designed to be 1–300 µL/h, which requires 30–45 min of pre-equilibration time, according to the condition of this study, to measure accurately the CO_2 evolution rate. If the CO_2 evolution rate is very low (≤ 5 µL/h), the headspace of the respirometer must be kept minimal to hasten the equilibration. The respirometer was designed so that the size of the reaction chamber stays the same while the size of the sample vial may vary according to the need of samples and the requirement of a minimal headspace.

The microrespiration was compared with an established IR respirometer on samples of various CO_2 evolution rates (Figure 6). The regression analysis indicates a very good 1:1 linear relationship (slope = 1.032 and $r^2 = 0.978$) between the 2 methods in a wide range (2–280 µL/h) of respiration rates.

One of the advantages of the microrespirometer is its ability to determine the CO_2 evolution rate accurately at the µL/h level in a short time. Determination of CO_2 evolution rates at a µL/h level is quite a challenge even for a sophisticated IR method. The IR analyzer must be able to detect less than 10 ppm (v/v) changes of CO_2 concentration with certainty during a period of hours. The accuracy of an IR analyzer method is further limited by the uncertainty of the volume occupied by a solid sample in most cases. Variation of headspace humidity, pressure, and temperature all affect the accuracy and precision of an IR respirometer. Because the microrespirometer method is based on the principle of CO_2 absorption–evolution equilibrium, its accuracy is not affected by headspace volume, humidity, pressure, or initial CO_2 concentration. The simplicity, noninstrumental nature, and very modest costs of the microrespirometer will make it available to many laboratory and field applications where accurate and rapid determination of respiration rate is required.

Conclusion

A novel noninstrumental microrespirometer was developed for the determination of CO_2 evolution rates. The microrespirometer is based on the simple principle of acid–base titration at a steady-state of CO_2 absorption/evolution. The microrespirometer is sensitive (detects µL CO_2/h), rapid (measurements made in 1 h), versatile (applicable to solid and liquid samples in a wide range of sizes), simple (in structure and operation), and economical (costs a fraction of an IR respirometer). The microrespirometer is suitable for monitoring real-time microbial activities in food, agricultural, and environmental samples, with regard to quality assurance and health safety concerns, under laboratory or field conditions.

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EXHIBIT B

MICROBIOLOGICAL METHODS

Real-Time Determination of Microbial Activity of Pasteurized Fluid Milk Using a Novel Microrespirometer Method

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The effectiveness of the rapid CO_2 evolution rate (CER) method was evaluated by using a novel noninstrumental microrespirometer to determine the microbial activity of pasteurized milk and comparing it with traditional culturing methods in homogeneous milk samples. Three different kinds of milk (skim, 1% fat, and whole) stored at 2 temperatures (4° and 7°C) were measured daily for CER, aerobic plate count (APC), and psychrotrophic bacterial count. The mean initial rates of CO_2 evolution for all 3 samples stored at the 2 temperatures ranged from 3.42 to 3.71 μL/h/mL and increased to 29 μL/h/mL and above on the final day of the experiment. Regression analysis showed a high correlation (R = 0.98–0.99) between the APC and CER results in combined milk samples. A cut-off value of CER (25 μL/h/mL) for milk spoilage at refrigeration temperatures was identified. The real-time CER method shows promise as a potential alternative to the traditional culture method.

Microbiological deterioration is the main problem limiting the shelf-life of pasteurized fluid milk due to its high water activity, neutral pH, and rich nutrient content. The spoilage microorganisms in pasteurized milk at refrigeration temperatures are generally accepted to be psychrotrophic bacteria (1, 2). Total aerobic plate counts (APC) have a long history of use as an effective means to monitor microbial activity of dairy products, although this method involves a great deal of time, labor, and materials (3, 4). Quality assurance of highly perishable food products, such as fluid milk, needs test methods that are simple, accurate, and economical, with speedy results available to allow a quick decision on possible risks.

Rapid bacterial assessment methods can be established based on a variety of properties of microbes, including their characteristic size and mass ranges, chemical composition, growth characteristics, specific enzyme activities, immunological activities, and metabolic activity, which is measured in terms of substrate consumption, rate of product formation, changes in pH value, and redox potential changes (5). Several systems and techniques have been proposed as alternatives to the APC method commonly used in the dairy industry, including the pyruvate, adenosine triphosphate (ATP), impedance, and microcalorimetric methods (4, 6). However, all these methods suffer from at least one disadvantage. For example, enzyme detection techniques are insensitive and can only be used when bacterial cell numbers are at least 10^5 CFU/mL. Thus, they are diagnostic rather than predictive (7).

The bacterial metabolism products include gases. Because carbon dioxide (CO_2) is a product of bacterial energy metabolism (respiration), detection of CO_2 evolution is a potential method for detecting and monitoring the growth of microorganisms (8–12). A simple and economical microrespirometer has been developed by Hsieh and Hsieh (13). This non-instrumental system for measuring the CO_2 evolution rate (CER), which is both simple to construct and easy to operate, is based on an alkaline trap and the acid-base titration principle. During bacterial growth, the CERs in either solid or liquid samples can be directly determined without the need for any sample pretreatment. This microrespirometer method has been successfully used to assess the microbial quality of solid perishable food, such as ground beef (8).

The objectives of this study were (*1*) to investigate the feasibility of using the microrespirometer to determine the microbial activity in a liquid sample system, i.e., pasteurized fluid milk samples at 2 refrigeration temperatures, over time; and (*2*) to validate the CER results obtained using the microrespirometer by comparing them with the 2 traditional bacterial enumeration methods of APC and psychrotrophic bacterial count (PBC).

Experimental

Chemical Reagents

Analytical grade NaOH, $BaCl_2$, phenolphthalein, ethanol, peptone water, and pH buffer standard were obtained from Fisher Scientific Co. (Fair Lawn, NJ). A 50 mL amount of 0.002N NaOH/$BaCl_2$ indicator solution was prepared daily by

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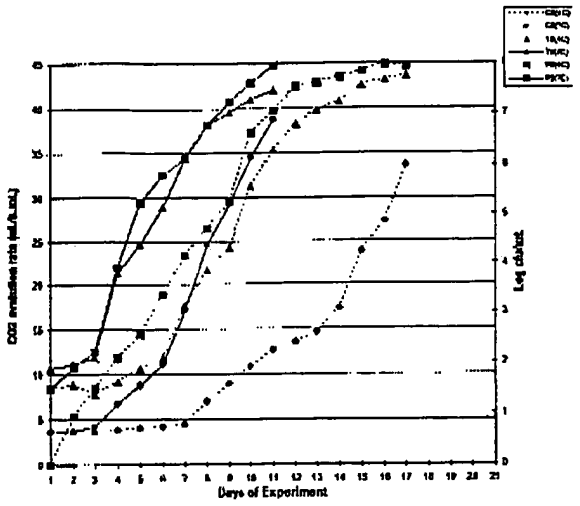

Figure 1. Relationship botween CER, bacterial counts (APC and PBC), and storage time of skim milk samples stored at 4° and 7°C. CS = CER of skim milk; TS = Total APC of skim milk; PS = PBC of skim milk. Standard deviations are within 0.0244–1.3591 from the moans (n = 6).

diluting 0.5 mL 0.2N NaOH, 1.0 mL 0.2N $BaCl_2$, and 0.5 mL indicator solution (0.5% phenolphthalein in 50% ethanol solution) with deionized water.

Milk Samples

Three types of milk samples (skim, 1% low fat, and whole milk with 3.25% milkfat) and 2 storage temperatures (4° and 7°C) were chosen to compare the correlation and sensitivity of the novel rapid method with the conventional methods for each of the 6 samples. To compare the results from the new rapid method with the traditional culturing method, one completely homogeneous sample was used. To ensure sample homogeneity and avoid further contamination of this highly perishable sample, all samples were taken from one sealed jug rather than pooling several jugs of each type of milk for all analyses under each storage condition.

Two of each type of pasteurized milk samples, packaged in half-gallon plastic jugs with the same brand and expiration date, were purchased from a local grocery store. They were immediately stored in refrigerators, with one set stored at 4°C and the other at 7°C until the end of the experiment. Each day, sensory examinations to detect the onset of spoilage were made immediately upon opening each original container of milk sample. About 30 mL of each milk sample was then aseptically transferred from the container into a 50 mL tube for measurement of CER, total bacterial count, and PBC. Samples for microbiological sampling were taken first from each tube before subsequent sampling for the determination of CER.

Bacterial Enumeration

Petrifilm™ SM Plates (Medical Products Division, 3M Company, St. Paul, MN) were used for enumeration of APC and PBC according to the manufacturer's instructions. A 1 mL amount of properly diluted (a series of 10-fold dilutions) milk sample was pipetted directly to the center of the prescribed plating area. Petrifilm plates were incubated at 32°C for 48 h for APC, and at 7°C for 10–15 days for PBC. All colonies were counted in the countable range (20–300 CFU). To compute the bacterial count, the total number of colonies per plate was multiplied by the reciprocal of the dilution used.

Determination of CER

The noninstrumental microrespirometer used in this study was designed by Hsieh and Hsieh (13). The 50 mL sterile tubes containing ca 30 mL milk from each sample were placed in a water bath shaker for 30 min at 25°C to bring the milk sample to room temperature. The milk samples were then bubbled with CO_2 free air, which was generated by an air pump that passed air through a strong alkaline solution trap (2N NaOH), for 10 min to remove the dissolved CO_2 ($DeCO_2$ treatment) in the milk. After the $DeCO_2$ treatment, 5 mL of each milk sample was placed in sample vials, which were then coupled in turn to the reaction chamber of the microrespirometer, and the assay was conducted according to Hsieh and Hsieh (13).

A 1 mL volume of 0.002N alkaline indicator solution was injected into the reaction chamber with a sterile disposable syringe. The microrespirometer was placed on a Platform Shaker (New Brunswick Scientific Classic Series, Edison, NJ) and shaken at a fixed rate of 240 rpm for 30–40 min to ensure adequate removal of the pre-existing CO_2 in the reaction chamber. The alkaline solution was then withdrawn, and 0.1 mL of the fresh alkaline solution was injected. The time needed to consume the 0.1 mL of alkaline solution, indicated by the color change from purplish red to faint pink, was recorded.

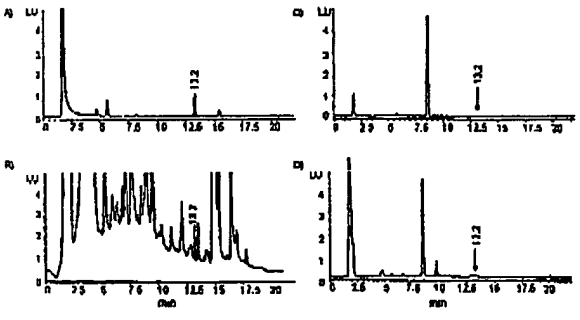

Figure 2. Relationship between CER, bacterial counts (APC and PBC), and storage time of 1% fat milk samples stored at 4° and 7°C. CF = CER of 1% fat milk; TF = Total APC of 1% fat milk; PF = PBC of 1% fat milk. Standard deviations are within 0.0199–3.3853 from the means (n = 6).

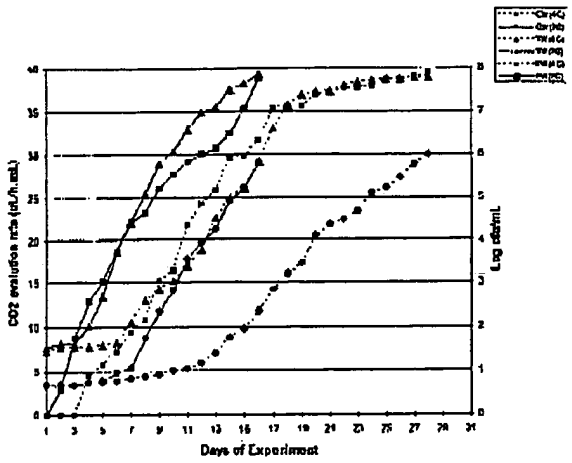

Figure 3. Relationship between CER, bacterial counts (APC and PBC), and storage time of whole milk samples stored at 4° and 7°C. CW = CER of whole milk; TW = Total APC of whole milk; PW= PBC of whole milk. Standard deviations are within 0.0203–1.0108 from the means (n = 6).

Injection of 0.1 mL increments of alkaline solution was repeated 3 times for each sample, and the time required to consume each increment of alkalinity was recorded. The average of the time required to consume the 0.1 mL alkaline solution was used to calculate the CER using the following formula (13):

$$\text{CER, } \mu\text{L/h/mL} = 22.4\ (0.1 \times 10^3 \times M/2) / [t/60]\ V$$

where M is the concentration of the alkaline solution in mole/L, 22.4 is the gaseous volume (mL) of 1 mole of CO_2 at a specific temperature, t is the time required to consume 0.1 mL alkaline solution in a minute, and V is the volume of the milk sample in mL.

Onset of Spoilage

The onset of spoilage of each milk sample was determined by daily sensory examination by the same 2 trained judges. Each day the original milk containers were removed from the refrigerator and the judges assessed the odor of each milk sample. Odors were judged on an intensity scale of 4 points: fresh odor, light off-odor/off-odor, and strong/bad off-odor. An off-odor that left a distinct smell was generally regarded as objectionable. Although some milk may develop bitter taste before a strong odor is detected, taste evaluation was not included in this study to avoid the possible risk of human exposure to high numbers of spoilage or pathogenic bacteria towards the end of the shelf-life. Agreement had to be reached between the 2 judges before the description of the odor could be recorded. Whenever there was any doubt concerning the presence of an objectionable odor, that particular sample was reassessed the next day for confirmation. The day the off-odor was confirmed represented the day the sample was determined unacceptable.

Statistical Analysis

All experiments were conducted in duplicate daily from day 1 (day of purchase) to spoilage. The correlation coefficients (R) were computed and compared using SPSS software (14) for all 3 methods: CO_2, APC, and PBC. Linear and quadratic regression analyses were conducted, also using SPSS, to quantitate the relationships between time of storage and CO_2 rate, APC, and PBC (15).

Results and Discussion

Bacterial Enumeration

The bacterial counts in milk samples were made daily until milk became spoiled, as determined by odor. Typical bacterial growth curves for skim, 1% fat, and whole milk samples stored at 4° and 7°C are shown in Figures 1, 2, and 3, respectively. **Fg 1-3**

On day 1, which was the first day of the experiment, the initial APCs in samples stored at 4°C were 1.49, 1.43, and 1.46 log CFU/mL for skim milk, 1% fat milk, and whole milk, respectively (Table 1). The initial APC in all 3 types of milk samples stored at both 4° and 7°C did not differ significantly ($p > 0.05$), with APC <100 CFU/mL. The initial APCs in samples stored at 7°C were 1.89, 1.72, and 1.54 log CFU/mL for skim milk, 1% fat milk, and whole milk, respectively (Table 1). Each milk sample (skim, 1% fat, and whole) underwent an increase in total APC during storage at both temperatures, and showed a typical growth profile with lag phase and log phase. The maximum counts in all milk samples at the point when milk was determined organoleptically to be unacceptable were between 10^7 and 10^8 CFU/mL. On the final day of the experiment, the APCs of milk samples stored at 4°C were 7.72, 7.98, and 7.78 log CFU/mL for skim milk, 1% fat milk, and whole milk, respectively (Table 1A), and for milk samples stored at 7°C, the APCs were 7.46, 7.89, and 7.85 log CFU/mL for skim milk, 1% fat milk, and whole milk, respectively (Table 1B).

The results of APCs in this experiment supported the previous findings (2, 16) that an APC reading of 10^7–10^8 CFU/mL in milk was the generally accepted number of microorganisms in milk that indicated that spoilage had occurred. Wilbey (17) suggested 10^7 CFU/mL as the threshold for detection of off-odor and bitterness in milk. In our experiment, a detectable off-odor for all milk samples was associated with high bacterial counts, typically in excess of 10^6 CFU/mL, and when the APC reached between 10^7 and 10^8 CFU/mL, all milk samples became unacceptable. **Tb1**

The PBC results in the experiments showed similar growth profile (Figures 1–3). The initial PBCs in all 3 milk samples were undetectable or very low, with PBC <50 CFU/mL. On day 1, log PBC was 0 for all milk samples at 4°C storage temperature (Table 1A). At 7°C storage temperature (Table 1B), the initial PBC was 1.49 log CFU/mL in skim milk, 1.70 log CFU/mL in 1% low fat milk, and 0 in whole

Table 1. Multiple comparative analyses of results of 3 methods on 3 experiment days for milk samples at 4° and 7°C ($P < 0.05$)

Experiment day	CER, μL/h/mL[a]			APC, log CFU/mL[b]			PBC, log CFU/mL[c]		
	Skim	1% Fat	Whole	Skim	1% Fat	Whole	Skim	1% Fat	Whole
				A: 4°C					
Day 1[d]	3.6198	3.6451	3.4418[e]	1.4914	1.4314	1.4624	0.000	0.0000	0.0000
Exp.Day[f]	6.9157[e]	4.8362[e]	4.3967[e]	3.8573[e]	2.7324	2.6232	4.6990[e]	4.1614[e]	2.1673[e]
End Day[g]	33.6084[e]	38.7505[e]	29.8261[e]	7.7243	7.9754[e]	7.7782	7.9294[e]	8.6794[e]	7.8663[e]
				B: 7°C					
Day 1	3.7134[e]	3.5247	3.4244	1.8976	1.7243	1.5441	1.4914[e]	1.7076	0.0000[e]
Exp.Day	24.6032[e]	18.1673[e]	8.7125[e]	6.7782[e]	7.0334[e]	5.0792[e]	6.7782	7.0000	4.6435[e]
End Day	38.7452[e]	33.0713[e]	28.9967[e]	7.4624[e]	7.8921	7.8513	7.9425[e]	8.0086[e]	7.7634[e]

[a] CER = CO_2 evolution rate.
[b] APC = Aerobic plate count.
[c] PBC = Psychrotrophic bacteria count.
[d] Day 1 = First day of the experiment.
[e] Mean difference is significant at $P < 0.05$
[f] Exp. day = Expiration day of the milk.
[g] End day = Day designated as the end of shelf-life for the milk.

milk. The PBC results were also in agreement with the previous studies of Henyon (18) and Bishop and White (19), who found that the initial PBCs in freshly pasteurized milk were absent or very low because most of the psychrotrophic bacteria cannot survive pasteurization. Dabbah et al. (20) indicated that the presence of psychrotrophic bacteria in pasteurized milk can usually be attributed to post-pasteurization contamination.

As shown in Figures 1–3, the psychrotrophic bacteria grew rapidly in milk after about 4 days of storage at both refrigeration temperatures, even though the initial count was absent or very low. At the end of the milk's shelf life, the maximum numbers of psychrotrophic bacteria had reached 10^8 CFU/mL. On the final day of the experiment, for milk samples stored at 4°C, log PBC was 7.93 for skim milk, 8.68 for 1% fat milk, and 7.87 for the whole milk (Table 1A). For milk samples stored at 7°C, log PBC on the last day of the experiment was 7.94 for skim milk, 8.01 for 1% fat milk, and 7.76 for whole milk (Table 1B).

These findings were also supported by many previous studies (18, 19, 21) that found that psychrotrophic bacteria grow fast at low temperatures and are the primary cause of spoilage in refrigerated pasteurized milk. Maxcy (22) reported that psychrotrophic bacteria account for 90% of the total bacterial population when milk spoil, and suggested that measurement of the total PBC could be an important method for determining post-pasteurization contamination of milk. However, the PBC method is not routinely used because of the long incubation time and low incubation temperature required.

CER Measurement

As bacterial counts increased, the CER also increased in all the milk samples during storage at both temperatures, with a small rate of increase in the lag phase and a large rate of increase in the log phase (Figures 1–3). The mean initial rates of CO_2 evolution for all 3 samples (skim milk, 1% fat milk, and whole milk) stored at the 2 temperatures ranged from 3.42 to 3.71 μL/h.mL (Table 1), and the CER in the whole milk sample was lower ($P < 0.05$) than that in either the skim milk or 1% fat milk samples.

On the final day of the experiment, for milk samples stored at 4°C, the CER was 33.61 μL/h/mL for skim milk, 38.75 μL/h/mL for 1% fat milk, and 29.83 μL/h/mL for whole milk (Table 1A). For milk samples stored at 7°C, the CER was 38.75 μL/h/mL for skim milk, 33.07 μL/h/mL for 1% fat milk, and 29.00 μL/h/mL for whole milk (Table 1B). The mean CER for skim milk and 1% fat milk was higher than that for the whole milk ($P < 0.05$).

Correlation Among CER, APC, and PBC

Correlation coefficients were computed among CER, APC, and PBC for the skim milk, 1% fat milk, and whole milk at both 4° and 7°C. High correlations were found between CO_2 and log APC, and between CO_2 and log PBC (Table 2). As shown in Table 2A, the correlation coefficient ($P < 0.01$) was 0.88 for the relationship between CER and APC, and 0.81 for the relationship between CER and PBC for the skim milk stored at 4°C. For the sample stored at 7°C, even higher correlations ($P < 0.01$) were found between CER and APC ($R = 0.92$), and PBC ($R = 0.89$). Table 2B shows that CER was

Table 2. Comparative analysis of (A) skim milk, (B) 1% fat milk, and (C) whole milk by Pearson correlation coefficient ($P < 0.001$)

	4°C			7°C		
Method	CER	APC	PBC	CER	APC	PBC
			A: Skim milk			
CER[a]	—	0.880	0.810	—	0.923	0.899
APC[b]	0.880	—	0.973	0.923	—	0.990
PBC[c]	0.810	0.973	—	0.899	0.990	—
			B: 1% Fat milk			
CER	—	0.899	0.903	—	0.928	0.861
APC	0.899	—	0.975	0.928	—	0.970
PBC	0.903	0.975	—	0.861	0.970	—
			C: Whole milk			
CER	—	0.933	0.867	—	0.934	0.910
APC	0.933	—	0.977	0.934	—	0.977
PBC	0.867	0.977	—	0.910	0.977	—

[a] CER = CO_2 evolution rate (μL/h/mL).
[b] APC = Aerobic plate count (log CFU/mL).
[c] PBC = Psychrotrophic bacteria count (log CFU/mL).

significantly correlated ($P < 0.01$) with both APC (R = 0.90) and PBC (R = 0.90) for the 1% milk stored at 4°C. Significant correlations ($P < 0.01$) were also found between CER and APC (R = 0.93) and CER and PBC (R = 0.86) for the sample stored at 7°C. Similar results were found in whole milk as displayed in Table 2C. The CER was significantly correlated ($P < 0.01$) with both APC (R = 0.93), and PBC (R = 0.93) for the whole milk stored at 4°C. Significant correlations ($P < 0.01$) were also found between CER and APC (R = 0.87) and PBC (R = 0.91) for the sample stored at 7°C. Tb2

In general, the values showed a higher degree of agreement between CER and APC than PBC for all the milk samples stored at both 4° and 7°C. Therefore, a quadratic regression analysis for the combined milk samples was used between log CER and log APC to confirm the findings of a high correlation between CER and APC, and to identify a suitable cut-off point for milk spoilage. The regression model showed that R was as high as 0.99 for milk stored at 4°C, which indicated that the 2 variables were highly related ($P < 0.0001$) such that as overall APC increased, the overall CER increased (Figure 3A). CER and APC for milk samples stored at 7°C were also highly related with R = 0.98 ($P < 0.0001$), as shown in Figure 3B. This study thus found that the results obtained using the APC and CER methods are highly congruent, and that either CER or APC could be used as a reliable indicator for bacterial growth in pasteurized milk. The results also suggest that the CER method is a possible alternative for the enumeration of bacterial numbers in pasteurized milk under routine laboratory conditions.

By extrapolation, a CER of 26 μL/h/mL was identified as the cut-off point for spoilage of pasteurized milk (APC >log 7 CFU/mL) stored at 4°C, and 27 μL/h/mL for milk stored at 7°C under our laboratory conditions. In our experiment, once the CER reached this cut-off value, a detectable odor was present in all the milk samples. Therefore, based on this result, a safe cut-off CER of <25 μL/h/mL should be considered to be the threshold of acceptance of commercial pasteurized milk. Because the CER method measures microbial metabolic activity, rather than cell numbers, the numbers of bacteria can only be estimated. In addition, the species, growth stages, sample matrixes, and total population of organisms present in the sample could all affect the metabolic activity of the microorganisms. The possible predominance of a specific microorganism cannot be distinguished by the CER method. However, in a previous study (8), a CER value of 25 μL/h/g was also empirically determined to be the safe cut-off value for acceptability of both fresh and previously frozen ground beef stored at different refrigeration temperatures (4° and 7°C). It appears that this CER value of 25 μL/h/g or 25 μL/h/mL could be used as a universal threshold of acceptance, regardless of the storage conditions, types of dominating microorganisms, and types of food.

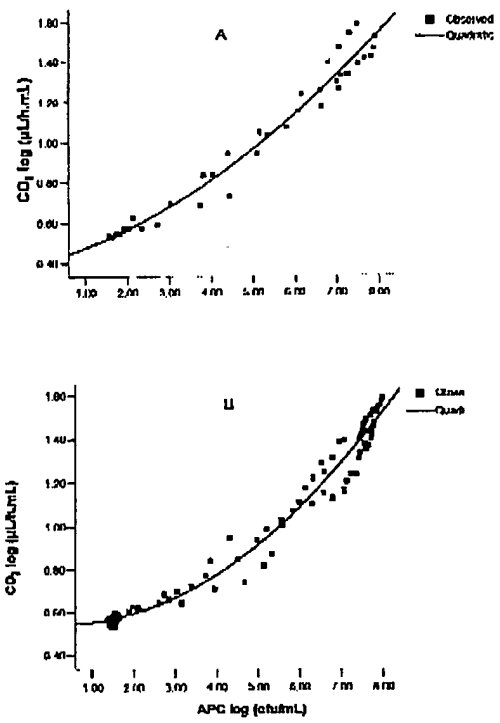

Figure 4. Quadratic relationship between log CER and log APC of all the milk samples stored at (A) 4°C, $Y = 0.5422 - 0.00649X + 0.01622X^2$, R = 0.9876; and (B) 7°C, $Y = 0.40507 + 0.05983X + 0.01042X^2$, R = 0.9842.

The application of the noninstrumental microrespirometer for CO_2 detection in this study has clearly revealed that this method has many advantages. It is comparable to conventional plating methods in determining the presence of microbial contamination and is easy to set up because preparation and sterilization of media are not required. The CER method is highly sensitive with the detection limit in the order of µL/h level. It does not require technical expertise beyond that necessary for proper execution of the other bacterial enumeration methods. A distinct advantage of the CER method over the APC method is that test results can be obtained within 1 h.

XXXAU: FIGURE 4 IS NOT CITED IN TEXT XXX

Conclusions

The results obtained by the CER method using a noninstrumental microrespirometer in this study were highly comparable with those obtained by traditional APC and PBC methods for all the milk samples (skim milk, 1% fat milk, and whole milk) at both storage temperatures (4° and 7°C) examined. High correlation coefficients between the CER and APC indicated that measurement of the CER was a viable alternative for determining microbial activity in pasteurized milk. A CER of 25 µL/h/mL was identified as the cut-off value for spoilage of pasteurized milk stored at refrigeration temperatures under laboratory conditions. The microrespirometer, therefore, is a rapid, sensitive, simple, economic, and nondestructive method that can be used to determine the real-time microbial quality of a fluid milk sample under both laboratory and field conditions at a very modest cost.

Acknowledgment

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EXHIBIT C

Comparison of the Real-time Microrespirometer and Aerobic Plate Count Methods for Determination of Microbial Quality in Ground Beef

X. LI AND Y.-H.P. HSIEH

ABSTRACT: Real-time microrespirometer measurements were compared with the aerobic plate count (APC) method to assess microbial quality of ground beef stored at 4 °C and 7 °C with and without previous freezing. The samples were monitored daily for CO_2 evolution rate (CER) using a microrespiromeler, APC, and were evaluated for color and odor changes by a sensory panel. The CER was highly correlated with the APC for all storage conditions (r^2 = 0.787 to 0.952). The onset of meat spoilage was more closely associated with a specific CER value (>25 μL/h/g) than APC. The new method was found to be more accurate in predicting meat spoilage, especially for previously frozen samples.

Keywords: carbon dioxide, respiration rate, microrespirometer, ground beef, spoilage

Introduction

In the U.S., the consumption of ground beef is very common. According to the Natl. Cattleman's Assn., the average consumption of retail ground beef for each American was 33.7 lb in 1980 and 28.8 lb in 2001, accounting for 44.1% and 43.2% of the total consumption of beef, respectively (Cattle-Fax and USDA 2002). However, ground beef is a highly perishable food because of its high water activity, near neutral pH, rich nutrient contents, and its large surface area that tends to support bacterial growth (Jay 2000). Although some muscle enzymes contribute to meat deterioration, microbial growth is the most important factor associated with the quality maintenance of fresh meat. Therefore, rapid and sensitive methods for microbial quality determination in ground meat are highly desired by the meat industry for quality control.

The conventional aerobic plate count (APC) method and several modified cultural methods (Chain and Fung 1991; Linton and others 1997) are relatively simple and are the most commonly used method for monitoring the microbial contamination in meat. These methods assess the number of viable organisms present, not their metabolism (Seymour and others 1994), and require at least 48 h of incubation to obtain a result. The U.S. Dept. of Agriculture (USDA) recommends that consumers keep beef either in a freezer or in a refrigerator immediately after purchase. Freezing can cause bacterial injury, even death, and although injured bacteria are able to form colonies on nonselective agar, they exhibit reduced metabolic activity after freezing and thawing (Ray and Speck 1973). Because the metabolic activity of spoilage bacteria is the major cause of meat deterioration, cultural methods may not be a reliable means of determining the microbial quality and predicting the shelf life of ground beef that has been stored at freezing temperatures.

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Carbon dioxide evolution is a common indicator of biological activity. Measurement of CO_2 evolved from microorganisms is one of the approaches that have often been used to assess the microbial load in food, agricultural, and clinical samples. On the basis of this principle, the instrument, BacT/Alert 3D has been commonly used in hospitals monitoring the sterility and bacterial growth in clinical specimen (Carricajo and others 2001; Krisher and others 2001). Measurement of CO_2 using gas chromatography (Guerzoni and others 1985; Basem and others 1992), radiometric techniques (Previte 1972), and an infrared CO_2 analyzer (Threlkeld 1982; Chew and Hsieh 1998; Hsieh and Hsieh 1998) have all been successful in determining the level of contamination by mesophilic and coliform bacteria in food samples, including hamburger, milk, water, catfish, and bottled juice. Recently, Hsieh and Hsieh (2000) developed a noninstrumental microrespirometer technique that is fast (<2 h), sensitive, and easy to operate. This technique measures the CO_2 evolution rate (CER) at the equilibrium point, where the CO_2 absorption rate equals the CER, and is based on acid-base titration. The new microrespirometer is suitable for both routine laboratory tests and for use in the field. So far, no studies have been reported using this method for the determination of the microbial quality in meat, for example, ground beef. The objectives of the present study were (1) to compare the effectiveness of the microrespirometer with the APC method for analyzing ground meat samples with respect to the prediction of spoilage; (2) to evaluate the sensitivity of these 2 methods in samples stored under 2 refrigeration temperatures, 4 °C and 7 °C, both with and without previous freezing; and (3) to establish a cut-off value of acceptability (the CER for the marginal state) for the microrespirometer method.

Materials and Methods

Sample collection and preparation

Locally purchased fresh ground beef chuck containing 20% fat was used as the sample in this study. The sample was transported to the laboratory on ice and prepared immediately after purchase

under aseptic conditions. To ensure complete homogeneity, the sample was reground 3 times and randomly divided into small portions (approximately 40 g). Each portion was transferred into a sterile sampling bag, sealed, and then pressed by hand to form a layer with a uniform thickness (approximately 1 cm). The packaged meat samples were stored under 6 different conditions: refrigeration at 4 °C, refrigeration at 7 °C, freezing at −22 °C for 10 d followed by refrigeration at 4 °C, freezing at −22 °C for 10 d followed by refrigeration at 7 °C, freezing at −22 °C for 20 d followed by refrigeration at 4 °C, and freezing at −22 °C for 20 d followed by refrigeration at 7 °C. From day 0 (either the day of purchase or the day a sample was removed from the freezer and placed in the refrigerator), 1 pack of sample was randomly selected from each storage treatment daily and tested by the APC, CER, and an assessment of the sensory characteristics, including color and odor, until a strong off-odor became evident.

Sensory evaluation

Five trained panelists evaluated the color and odor of meat samples daily throughout the study. Color was evaluated under normal lighting with the samples in intact packs placed on a white background. A 5-point structured scale with 1-digit intervals was used to evaluate the meat color. The scale used was as follows: 1 = bright red; 2 = red; 3 = slightly brown; 4 = moderately brown; and 5 = extremely brown (NLSMB 1991). Samples with scores of 3 and below are regarded as acceptable. The same panelists evaluated the odor by sniffing each sample once the package was opened. The scale used for odor appraisal was as follows: 1 = fresh; 2 = meaty; 3 = not so fresh; 4 = stale; and 5 = extremely bad (Sorheim and others 1999). Samples with scores of 3 or below are considered acceptable. The experiment continued until very strong off-odors became evident.

APC

Ten grams of each ground beef sample were accurately weighed and added to sterile 0.1% peptone water (Difco, Sparks, Md., U.S.A.) to make a 1:9 dilution (wt:vol). Each sample was then blended and serial dilutions were made with the sample blends. Total APC was determined using Petrifilm (3M Microbiology Products, St. Paul, Minn., U.S.A.) according to the manufacturer's instructions. Enumeration of colonies was performed after incubation at 35 °C for 48 h (Ingham and Moody 1990).

Measurement of CER

The CER of the ground beef samples was determined by using a microrespirometer according to Hsieh and Hsieh (2000). The microrespirometer consists of a spherical reaction chamber and a sample vial. Ground beef samples (about 8 g) from each storage treatment were accurately weighed and placed in separate 25-mL sterile sample vials (Fisherbrand EPA vials, Fisher Scientific, Fair Lawn, N.J., U.S.A.). The reaction chamber and the sample vial were then coupled to form a closed headspace. One milliliter of freshly prepared 0.002 *M* alkaline indicator solution (diluting a mixture of 1 mL of 0.2-*N* NaOH, 2 mL of 0.2-*N* $BaCl_2$, and 1 mL of 0.5% phenolphthalein indicator with deionized water to 100 mL) was injected into the reaction chamber using a disposable syringe. The loaded microrespirometer was shaken on a horizontal shaker (New Brunswick Scientific C2 Platform, Edison, N.J., U.S.A.) at 240 rpm for 1 h—the pre-equilibration period to remove the existing CO_2 in the system. Upon reaching equilibrium, the remaining alkaline solution in the reaction chamber was withdrawn, and 0.2 mL of fresh alkaline indicator solution was immediately injected into the chamber using a syringe, after which shaking was resumed. The time required for the color to change from deep pink to faint pink was recorded. Injections of 0.2 mL alkaline solution were repeated until a consistent time was observed for the change. The average time required to consume the incremental 0.2 mL alkaline solution was used to calculate the rate of CO_2 evolution by applying the following formula:

$$\text{CER}\ (\mu\text{L/h/g}) = [(0.2 \times 10^3 \times M/2) \times 22.4] \,/\, (t[\text{min}]/60)/g$$

where M = the concentration of the alkaline solution; t = the time to convert the alkaline solution from a deep to a faint pink color; and g = the weight of the ground beef sample.

Table 1—The 1st day of meat spoilage based on meat color and odor evaluation under 6 different storage conditions

Storage conditions[a]	1st day of color score above 3 (score ± SD)[b]	1st day of odor score above 3 (score ± SD)[b]
4 °C	4 (4 ± 0)	5 (3.75 ± 0.5)
7 °C	3 (3.4 ± 0.55)	4 (3.75 ± 0.5)
Fr-10d-4 °C	7 (3.8 ± 0.45)	8 (3.8 ± 0.45)
Fr-10d-7 °C	4 (3.7 ± 0.58)	6 (4 ± 0)
Fr-20d-4 °C	6 (3.6 ± 0.55)	9 (3.8 ± 0.45)
Fr-20d-7 °C	5 (3.8 ± 0.45)	6 (4 ± 0)

[a]Fr-10d-4 °C = held at 4 °C after 10-d frozen storage; Fr-10d-7 °C = held at 7 °C after 10-d frozen storage; Fr-20d-4 °C = held at 4 °C after 20-d frozen storage; Fr-20d-7 °C = held at 7 °C after 20-d frozen storage. Samples with a score of color or odor above 3 were considered spoiled and thus unacceptable.
[b]n = 6.

Statistical analysis

All determinations were performed in duplicate. The entire experiment was repeated twice. The effects of refrigeration temperature (4 °C compared with 7 °C), frozen compared with nonfrozen storage, and the duration of frozen time (10 d compared with 20 d) on the APC and CER were analyzed by Student's *t*-test and 2-way analysis of variance at $\alpha = 0.05$ (SAS Inst. 1992). Simple linear and quadratic regression analyses using the Excel program (Microsoft Co., Seattle, Wash., U.S.A.) were used to determine the relationship between the CER values (log μL/h/g) and APC numbers (log colony-forming units [CFU]/g). Cut-off values for acceptability were obtained from a regression equation based on the sensory-evaluation results and references for proposed microbiological standards of meat.

Results and Discussion

Sensory characteristics

The sensory results are summarized in Table 1. The rate of discoloration was observed to be faster than that of odor changes for a given sample, with the color score reaching an unacceptable level 1st. For fresh ground beef samples, color scores above 3 occurred at day 4 at 4 °C and day 3 at a 7 °C storage temperature, whereas the meat odors for these samples did not become unacceptable until day 5 and day 4, respectively. Similarly, for previously frozen samples, the brown color was not apparent until day 7 and day 4 for samples stored at 4 °C and 7 °C, respectively, after 10 d of frozen storage. The brown color was not apparent until day 6 and day 5 for samples stored at 4 °C and 7 °C, respectively, after 20 d of frozen storage. The unacceptable odors were, however, detected at day 8 and day 6 for samples stored at 4 °C and 7 °C, respectively, after 10 d of frozen storage. The unacceptable odors were detected at day 9 and day 6 for samples stored at 4 °C and 7 °C, respectively, after 20 d of frozen storage. The occurrence of unacceptable meat color was usually 1 to 3 d earlier than the unacceptable meat odor. The discoloration of meat is mainly because of the oxidation of myoglobin

Table 2—The CO_2 evolution rates (μL/h/g) and aerobic plate counts (log CFU/g) of ground beef samples[a]

Storage conditions[b]	CO_2 evolution rate (μL/h/g)			Aerobic plate counts (log CFU/g)		
	Day 0	Onset of spoilage	End point	Day 0	Onset of spoilage	End point
4 °C	6.63 ± 0.16d	30.97 ± 1.20gi	46.34 ± 2.23j	3.81 ± 0.0071n	6.00 ± 0.014o	6.93 ± 0.035q
7 °C	6.63 ± 0.16d	29.24 ± 1.30fg	73.23 ± 2.57k	3.75 ± 0.0071n	5.96 ± 0.021o	6.26 ± 0.0071p
Fr-10d-4 °C	5.00 ± 0.18c	24.70 ± 0.62e	55.73 ± 3.01j	3.72 ± 0.021n	7.28 ± 0.028r	8.00 ± 0.014t
Fr-10d-7 °C	4.97 ± 0.17c	29.51 ± 0.62gh	105.66 ± 2.77l	3.69 ± 0.035n	7.55 ± 0.0071s	8.32 ± 0.021u
Fr-20d-4 °C	3.66 ± 0.01a	28.57 ± 1.66f	73.91 ± 4.91k	3.79 ± 0.092n	7.41 ± 0.084r	8.27 ± 0.028u
Fr-20d-7 °C	4.28 ± 0.16b	32.58 ± 1.11i	129.50 ± 4.98m	3.76 ± 0.035n	7.64 ± 0.028s	7.68 ± 0.071s

[a]Means within columns with no common letters (a through u) differ significantly ($P < 0.05$).
[b]4 °C = stored at 4 °C, 7 °C = stored at 7 °C; Fr-10d-4 °C = held at 4 °C after 10-d frozen storage; Fr-10d-7 °C = held at 7 °C after 10-d frozen storage; Fr-20d-4 °C = held at 4 °C after 20-d frozen storage; Fr-20d-7 °C = held at 7 °C after 20-d frozen storage

into metmyoglobin and is not necessarily caused by bacterial behavior (Gill 1996). However, the development of off-odors does result from the microbial metabolism of amino acids (Borch and others 1996). Thus, odor is a more accurate indicator of meat spoilage than color. According to the color and odor changes, the samples which had undergone a previous freezing treatment had a longer shelf life compared with those which had not been frozen (Table 1).

The bacterial growth curves for the meat samples are shown in Figure 1. The APC values on day 0, at the onset of spoilage, and at the experimental end-point are listed in Table 2. The APC values increased during storage for all the temperature conditions. On day 0, the initial bacterial loads for all 6 different storage conditions ranged from 3.67 to 3.81 log CFU/g and did not differ significantly ($P > 0.1$) (Table 2). This result was in agreement with a previous study, which reported that the initial bacterial load of ground beef was between 3.0 and 4.0 log CFU/g (Borch and others 1996). The USDA reported that the baseline of initial mesophilic bacteria in ground beef was 3.9 log CFU/g (Dormedy and others 2000).

The samples at the onset of spoilage had total bacterial counts of 6.00 log CFU/g and 5.96 log CFU/g for samples refrigerated at temperatures of 4 °C and 7 °C, respectively, and ranged from 7.28 log CFU/g to 7.64 log CFU/g for the 4 previously frozen samples. When the samples were determined to be completely spoiled, the APC values reached 6.93 log CFU/g and 6.26 log CFU/g for fresh samples stored at 4 °C and 7 °C, respectively, but ranged from 7.68 log CFU/g to 8.32 log CFU/g for previously frozen samples. These results supported the findings of previous studies (Carl 1975; Pivnick and others 1976; Mates 1983; ICMSF 1986; Jay 2000) that proposed an APC value of between 6.0 and 7.70 log CFU/g as the upper limit of acceptability in commercial fresh and frozen ground beef.

A significantly higher number for the APC ($P < 0.001$) of pre-frozen samples compared with that of nonfrozen samples was consistently observed at the onset of meat spoilage. There was an approximately 1.5 log difference in the APC values between the frozen and nonfrozen samples (Table 2). This difference can be explained by the reduced metabolism of some microorganisms injured during freezing and frozen storage, thus a higher number of spoilage bacteria must be attained over a longer storage time to produce enough metabolic products before the spoilage can be detected organoleptically. Some of the microorganisms are damaged by freezing because of the formation of ice crystals and exposure to the concentrated solute. This kind of damage is called metabolic injury because bacteria are still able to form colonies in complete nutrient media but not in selective media because of reduced metabolic activity (Ray and Speck 1973).

Presently, no clear microbiological standard of acceptability of ground beef has been established in the United States. The studies that associated the deterioration with sensory characteristics reported different bacterial counts corresponding to the onset of meat spoilage. Lambert and others (1991) observed that off-odor and slime were detected on meat when bacterial numbers reached 7.0 to 8.0 log CFU/cm², whereas Raccach (1998) found the range to be from 7.0 to 9.0 log CFU/cm². The Health Protection Branch of Health and Welfare Canada recommended that different microbiological criteria be applied to meat in acceptability ranging from log CFU/g and 7.0 log CFU/g, respectively, based on a national survey conducted between 1974 and 1975 (Pivnick and others 1976). Therefore, total bacterial number may not be an accurate indication of the meat quality, especially for previously frozen meat.

CO_2 evolution rates

As bacterial counts increased, CER also increased during storage under all experimental conditions, with a small increase in the lag phase and a pronounced increase in the log phase (Figure 2). The mean initial rates of CO_2 evolution for the 6 temperature treatments ranged from 3.66 to 6.63 μL/h/g (Table 1), and the CERs of

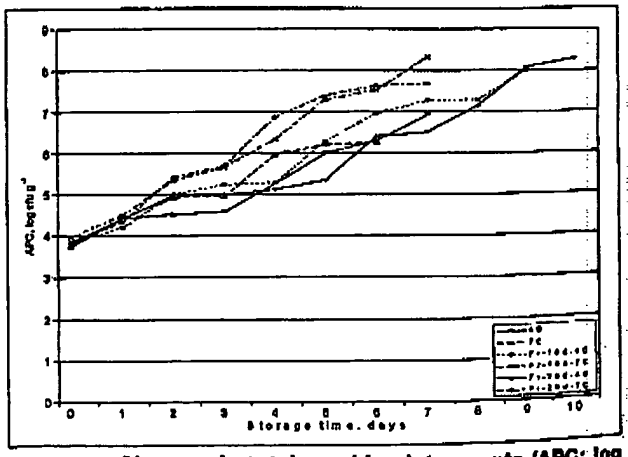

Figure 1—Changes in total aerobic plate counts (APC; log CFU/g) compared with storage time (d) under different storage conditions for ground beef samples. 4C = stored at 4 °C; 7C = stored at 7 °C; Fr-10d-4C = held at 4 °C after 10-d frozen storage; Fr-10d-7C = held at 7 °C after 10-d frozen storage; Fr-20d-4C = held at 4 °C after 20-d frozen storage; Fr-20d-7C = held at 7 °C after 20-d frozen storage. Standard deviations of APC are within 0.0071 to 0.613 of the means ($n = 2$).

Table 3—Effect of freezing on CO_2 evolution rates (μL/h/g) and aerobic plate count (APC) (log CFU/g) at 4 °C and 7 °C

		Day 1		Day 4		Day 6	
Parameter	Treatment	4 °C	7 °C	4 °C	7 °C	4 °C	7 °C
CO_2 evolution rate (μL/h/g)	Frozen	3.12 ± 0.21a	3.22 ± 0.29a	3.78 ± 0.31b	7.79 ± 2.28e	8.37 ± 0.74f	28.09 ± 4.39h
	Nonfrozen	7.04 ± 0.55c	7.58 ± 0.37d	25.97 ± 3.87g	28.15 ± 1.47h	41.27 ± 3.88i	62.81 ± 12.48j
APC (log CFU/g)	Frozen	4.12 ± 0.24k	4.37 ± 0.10k	5.39 ± 0.29l	6.59 ± 0.25n	6.58 ± 0.32n	7.80 ± 0.23p
	Nonfrozen	4.36 ± 0.08k	4.41 ± 0.04k	5.62 ± 0.29l	6.06 ± 0.13m	6.67 ± 0.22n	7.02 ± 0.20o

aMeans within columns with no common letters (a through p) differ significantly ($P < 0.05$).

the 4 frozen-treated groups were lower ($P < 0.05$) than those of the 2 refrigerated-only groups. In general, the CERs (μL/h/g) were approaching 30 μL/h/g when odors of spoilage became detectable, indicating similar metabolic activity levels at the onset of spoilage In all the samples for all the temperature treatments. On the last day of experimentation for each temperature treatment, the CERs were 46.34 μL/h/g and 73.23 μL/h/g for unfrozen samples refrigerated at 4 °C and 7 °C, respectively. For previously frozen samples, the CERs were as follows: 55.73 μL/h/g (frozen 10 d, then held at 4 °C), 105.66 μL/h/g (frozen 10 d, then held at 7 °C), 73.91 μL/h/g (frozen 20 d, then held at 4 °C), and 129.50 μL/h/g (frozen 20 d, then held at 7 °C), respectively (Table 3). The wide range of their final CERs suggests that different storage temperature conditions substantially influence bacterial metabolic activity.

Influence of refrigeration temperature

It is well established that a close relationship exists between the storage temperature and shelf life of the meat samples (Gill and Newton 1977; Greer and Jeremiah 1980). Results from our study clearly demonstrated that the refrigeration temperature is an important factor affecting the rate of the change of sensory characteristics, microbial growth, and respiration rate, and hence, the shelf life of the meat. In general, samples refrigerated at 4 °C showed a slower change in off-odor development and discoloration than those refrigerated at 7 °C, whether with or without previous freezing (Table 1).

Comparison of the 2 refrigerated-only samples shows that the average CERs were significantly different ($P < 0.05$), with samples stored at 7 °C having higher values than those stored at 4 °C for the same storage times; the APC values demonstrated a similar pattern, except for day 1 and day 6. When analyzing the data for the subsequent refrigeration temperatures of the pre-frozen samples, significant differences ($P < 0.05$) were also found between 4 °C and 7 °C for the same storage times, both in the CER and in the APC results (data not shown). Thus, these results suggest that both the microrespirometer method and APC method are sensitive to the effect of refrigeration temperature and are accurate ways to measure the deterioration of ground beef during refrigeration storage.

Influence of freezing and freezing length

An interesting finding was observed when comparing the CER and APC results between previously frozen samples and nonfrozen samples. Three experimental days (days 1, 4, and 6) were chosen for the comparison, each of which represented a different stage of meat ranging from freshness to spoilage. Data were analyzed using 2-way analysis of variance after classification into 4 groups in terms of frozen or nonfrozen treatment and 2 different refrigeration temperatures. It was found that there was a significant difference in CER between the frozen and nonfrozen samples for either 4 °C or 7 °C of refrigeration ($P < 0.05$); however, no significant difference ($P > 0.05$) was observed in APC values between the frozen and nonfrozen samples on the 3 selected experimental days, except for day 4 and day 6 for samples stored at 7 °C (Table 3). These results clearly demonstrate that freezing influences the microbial activity in ground beef samples, which can be sensitively reflected by the rates of CO_2 evolution, but not the APC results under laboratory conditions. The differences between the frozen and nonfrozen samples in terms of APC and CER results may be attributed to the different principles underlying these 2 methods. The APC method is based on the enumeration of colonies, and this method assumes that each colony develops from a single microbial cell of the original culture, whereas the microrespirometer method measures the overall CER produced by microbial metabolism. The sensory results showed that the off-odors were detected much later in the previously frozen samples than in the nonfrozen samples and the onset of spoilage appears to be more closely associated with a high CER value than with viable cell numbers. Therefore, the results from this study suggest that the microrespirometer method is a more sensitive method for the determination of microbial quality and would thus be more accurate for prediction of shelf life in meat samples.

The effects of 2 different freezing lengths (10 d compared with 20 d) on APC values and CERs were analyzed by factorial analysis. No significant difference in either the APC values ($P > 0.6172$) or the CER ($P > 0.5148$) was obtained between the 10-d frozen storage and

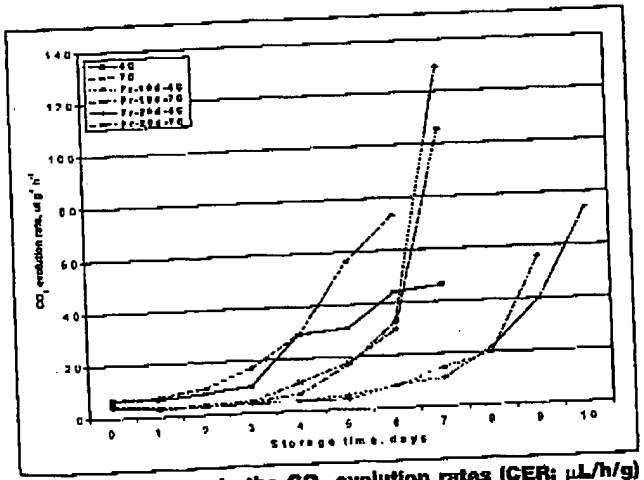

Figure 2—Changes in the CO_2 evolution rates (CER; μL/h/g) compared with storage time (d) under different storage conditions for ground beef samples. 4C = stored at 4 °C; 7C = stored at 7 °C; Fr-10d-4C = held at 4 °C after 10-d frozen storage; Fr-10d-7C = held at 7 °C after 10-d frozen storage; Fr-20d-4C = held at 4 °C after 20-d frozen storage; Fr-20d-7C = held at 7 °C after 20-d frozen storage. Standard deviations of CER are within 0.01 to 4.98 of the means ($n = 2$).

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20-d frozen storage samples. Some studies (Ray and Speck 1973; Raccach and others 2002) have reported that with an increased length of frozen storage, the bacterial population further decreases because of the recrystallization of small ice crystals and prolonged exposure to the concentrated extracellular and intracellular solutes. In this study, the 2 freezing durations tested at −22 °C may have been too close to observe such a difference.

Correlation between CER and APC values

With respect to the nonfrozen samples, the linear regression model showed the best fitting performance, $r^2 = 0.857$, for samples stored at 4 °C, which indicates that these 2 parameters were highly correlated. As overall APC increased, the overall CER also increased (Figure 3). A slightly lower correlation coefficient of 0.787 was obtained between the CER and APC values for samples stored at 7 °C. Amplification of the difference between the 2 methods because of more rapid microbial growth and increased activities at the higher storage temperature may be the reason for the lower correlation compared with that of samples stored at 4 °C.

However, when the linear regression model was used to relate the CER and APC of the pre-frozen samples, the fitting performance was not ideal, with the correlation coefficients ranging between 0.695 and 0.838 (data not shown). In contrast, a quadratic regression model obtained a higher correlation. With regard to the samples stored at 4 °C and 7 °C after a 10-d frozen storage period, high positive correlations with r^2 of 0.952 and 0.942, respectively, were obtained (Figure 4a and 4b). For samples stored at 4 °C and 7 °C after 20-d frozen storage, the correlation coefficients between the CER and APC were 0.899 and 0.847, respectively (Figures 4c and 4d). Different regression models between the nonfrozen and frozen samples suggest that there was a slower increment in the CER of the frozen samples compared with that of nonfrozen samples during storage, although no difference in total bacterial numbers (that is, APC values) between nonfrozen and frozen samples during storage was observed. Overall, the results of this study indicate that the APC and CO_2 evolution methods are very comparable, and CER could safely be used as a reliable indicator of bacterial activity in ground beef. In fact, the real-time microrespirometer method is more accurate than the APC method in predicting the spoilage of ground beef.

Determination of a cut-off value for CER

Once the spoilage odor became detectable, the APC levels of the 2 refrigerated-only samples were close to 6.00 log CFU/g (Table 2). This number is consistent with the upper limit for the acceptability of nonfrozen meat proposed by Pivnick and others (1976). Thus, by extrapolation, CERs of 26.98 μL/h/g and 28.21 μL/h/g, corresponding to APC = 6.0 log CFU/g, were identified as being suitable values to signal the onset of spoilage for fresh ground beef stored at either 4 °C or 7 °C under our laboratory conditions (Table 4). The values of the CER at the onset of spoilage for previously frozen samples were calculated, based on their corresponding regression equations, individually. In addition, based on the results of our sensory evaluation and the proposed level of spoilage for frozen samples (Mates 1983), an APC value of 7.5 log CFU/g was used to calculate the marginal value of the CER. The corresponding CER

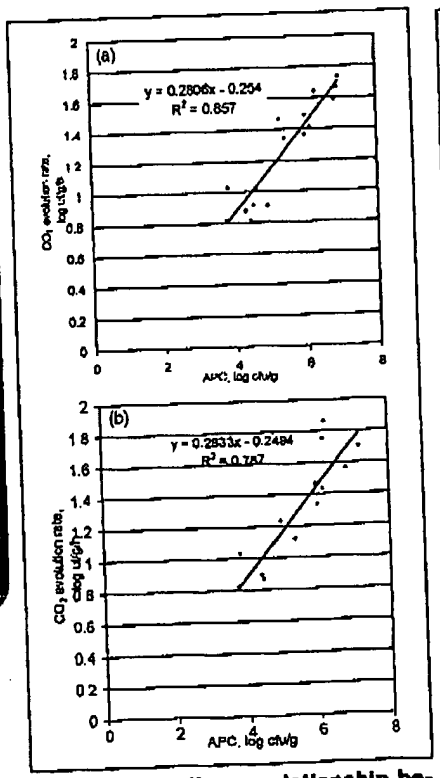

Figure 3—The linear relationship between the CO_2 evolution rate (CER; log μL/h/g) and aerobic plate counts (APC; log CFU/g) of the combined ground beef samples stored at a = 4 °C; and b = 7 °C.

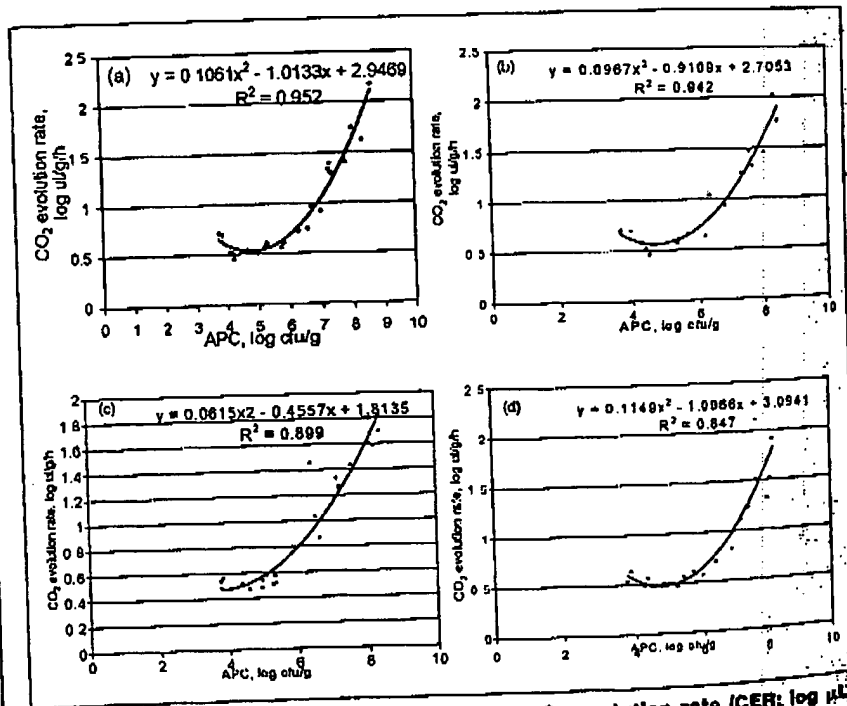

Figure 4—The quadratic relationship between CO_2 evolution rate (CER; log μL/h/g) and aerobic plate counts (APC; log CFU/g) of the combined ground beef samples. a = held at 4 °C after 10-d frozen storage; b = held at 7 °C after 10-d frozen storage; c = held at 4 °C after 20-d frozen storage; and d = held at 7 °C after 20-d frozen storage.

ranged from 26.49 μL/h/g to 29.04 μL/h/g (Table 4). Therefore, based on the results, a safe cut-off CER value of 25 μL/h/g should be considered as the threshold for the acceptance of ground beef.

Conclusions

Both the real-time (<2 h) microrespirometer method monitoring CER and the traditional APC cultural method can effectively assess the microbial quality in ground beef samples. Freezing influences the microbial activity in ground beef samples, which can be sensitively reflected by the rates of CO_2 revolution, but not the APC results under the experimental conditions. As the APC values associated with the onset of spoilage for nonfrozen and frozen ground beef samples varies significantly, that is, 6.0 log CFU/g and 7.5 log CFU/g, respectively, a CER value of 25 μL/h/g was identified to be the safe cut-off value for acceptability of ground beef stored at different refrigeration temperatures (4 °C and 7 °C), regardless of previous freezing. Thus, the microrespirometer method is not only more rapid but also more sensitive and more accurate than the conventional APC method in predicting the microbial quality of meat samples, especially for previously frozen samples. The use of the microrespirometer methods can be extended to other perishable meat products for real-time detection and monitoring of microbial contamination.

Acknowledgment

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Table 4—CO_2 evolution rates (μL/h/g) and aerobic plate count (APC) values at onset of spoilage

Temp. treatment[a]	Regression equation	APC value (CFU/g)	CO_2 evolution rate (μL/h/g)
4 °C	$Y = 0.2806X - 0.254$	1×10^6	26.89
7 °C	$Y = 0.2833X - 0.2484$	1×10^6	28.21
Fr-10d-4 °C	$Y = 0.1061X^2 - 1.0133X + 2.9469$	$1 \times 10^{7.5}$	27.23
Fr-10d-7 °C	$Y = 0.0967X^2 - 0.9109X + 2.7053$	$1 \times 10^{7.5}$	26.49
Fr-20d-4 °C	$Y = 0.0651X^2 - 0.4557X + 1.3135$	$1 \times 10^{7.5}$	28.25
Fr-20d-7 °C	$Y = 0.1149X^2 - 1.0966X + 3.0941$	$1 \times 10^{7.5}$	29.04

[a]X = log aerobic plate count (APC); Y = log CO_2 evolution rate; Fr-10d-4 °C = held at 4 °C after 10-d frozen storage; Fr-10d-7 °C = held at 7 °C after 10-d frozen storage; Fr-20d-4 °C = held at 4 °C after 20-d frozen storage; Fr-20d-7 °C = held at 7 °C after 20-d frozen storage.

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